

## DESCRIPTION

### TRANSGLUTAMINASE-PRODUCING STRAIN

#### 5 TECHNICAL FIELD

The present invention relates to a strain producing transglutaminase derived from actinomycetes and a process for producing transglutaminase by using the strain.

#### BACKGROUND ART

10 Transglutaminase is an enzyme which catalyzes an acyl transfer reaction of a  $\gamma$ -carboxy amide group of glutamine residue in a peptide chain. In particular, transglutaminase induces intramolecular or intermolecular formation of  $\epsilon$ -( $\gamma$ -Gln)-Lys cross linking to an  $\epsilon$ -amino group of a lysine residue in a protein. The enzyme has been widely used for processing proteins by using this property in the fields of food  
15 and medicine.

Since a long time ago, animal-derived transglutaminase has been known. It has been reported that transglutaminase has been found in animals, for example, in the liver of guinea pigs and in organs and blood of mammals (Connellan et al., Journal of  
20 Biological Chemistry, vol. 246, No. 4, pp. 1093-1098 (1971)), Folk et al., Advances in Enzymology, vol. 38, pp. 109-191 (1973); and Folk et al., Advances in Protein Chemistry, vol. 31, pp. 1-133 (1977)), and its enzymological properties have been studied. On the other hand, a different type of transglutaminase, which is not dependent upon calcium ( $\text{Ca}^{2+}$ ) and is therefore different from the above-mentioned  
25 animal-derived transglutaminase, has been found. Specifically, transglutaminase has been isolated and identified from *Streptomyces mobaraensis* [old name: *Streptoverticillium mobaraense*] IFO 13819 (Japanese Patent Unexamined Publication No. S64-27471), *Streptomyces griseocarneus* [old name: *Streptoverticillium griseocarneum*] IFO 12776, and *Streptomyces cinnamoneus* [old name:  
30 *Streptoverticillium cinnamoneum*] IFO 12852 (Japanese Patent Unexamined Publication No. 2001-186884), etc.

#### DISCLOSURE OF INVENTION

Conventionally, since transglutaminase has been produced via extraction and  
35 isolation, etc. from animals, micro-organisms, and the like, that are present in nature,

there have been many problems to be solved such as a low production yield and an expensive production cost, etc. Meanwhile, as to transglutaminase derived from micro-organisms, process for producing by using genetic recombination has been intensively studied. However, according to the first report of a process using genetic recombination (Biosci. Biotech. Biochem., 58,82-87 (1994), Japanese Patent Unexamined Publication No. H5-199883), the production amount was about 0.1 mg/l, which was far from industrial production level. Furthermore, according to the recent report (Japanese Patent Unexamined Publication No. 2001-186884), although the production level was improved to some extent, two-week microbial culture resulted in the productivity of only about 40 to 50 mg/l. Also, in this case, it cannot be said that sufficient productivity is obtained.

The present invention was made on the basis of the above-mentioned background, and the object of the present invention is to provide a strain capable of producing transglutaminase at a high efficiency and a process for producing transglutaminase using the strain.

In order to solve the above-mentioned problems, the present inventors have eagerly investigated. That is to say, for expressing transglutaminase derived from actinomycetes, the present inventors have investigated the combination of a transglutaminase structural gene, a promoter, a vector and host actinomycetes. As a result, the present inventors have succeeded in obtaining transformant having extremely high productivity of transglutaminase and have completed the present invention. The present invention provides the following configurations.

[1] A transformant of *Streptomyces mobaraensis*, comprising a structural gene of transglutaminase derived from *Streptomyces mobaraensis* and a promoter and a terminator acting on the structural gene, which are externally introduced.

[2] The transformant of *Streptomyces mobaraensis* according to [1], wherein the promoter is a promoter of transglutaminase derived from *Streptomyces mobaraensis*.

[3] The transformant of *Streptomyces mobaraensis* described in [1] or [2], wherein the terminator is a terminator of transglutaminase derived from *Streptomyces mobaraensis*.

[4] The transformant of *Streptomyces mobaraensis* described in any of [1] to [3], wherein the structural gene comprises a sequence set forth in SEQ ID NO: 1 or a

sequence obtained by modifying a part of the sequence, the sequence encoding transglutaminase.

5 [5] A transformant of *Streptomyces mobaraensis* comprising a DNA fragment having an externally introduced sequence set forth in SEQ ID NO: 2 or sequence obtained by modifying a part of the sequence, the sequence encoding transglutaminase.

10 [6] The transformant of *Streptomyces mobaraensis* described in any of [1] to [5], which is a transformant of *Streptomyces mobaraensis* S-8112 or a mutant strain thereof.

[7] A process for producing transglutaminase, comprising the steps of:  
culturing transformant of *Streptomyces mobaraensis* comprising a structural gene of transglutaminase derived from *Streptomyces mobaraensis* and a promoter and a  
15 terminator acting on the structural gene, which are externally introduced, under the conditions where the structural gene can be expressed; and  
collecting the produced transglutaminase.

20 [8] The process for producing transglutaminase described in [7], wherein the promoter is a promoter of transglutaminase derived from *Streptomyces mobaraensis*.

[9] The process for producing transglutaminase described in [7] or [8], wherein the terminator is a terminator of transglutaminase derived from *Streptomyces mobaraensis*.

25 [10] The process for producing the transglutaminase described in any of [7] to [9], wherein the structural gene comprises a sequence set forth in SEQ ID NO: 1 or a sequence obtained by modifying a part of the sequence, the sequence encoding transglutaminase.

30 [11] The process for producing transglutaminase described in any of [7] to [9], wherein the transformant of *Streptomyces mobaraensis* comprises a DNA fragment having an externally introduced sequence set forth in SEQ ID NO: 2 or sequence obtained by modifying a part of the sequence, the sequence encoding transglutaminase.

35 [12] The process for producing transglutaminase described in any of [7] to [11],

wherein the transformant of *Streptomyces mobaraensis* is a transformant of *Streptomyces mobaraensis* S-8112 or a mutant strain thereof.

5 [13] A transformant of *Streptomyces lividans* comprising a structural gene of transglutaminase derived from *Streptomyces mobaraensis*, and a promoter and a terminator acting on the structural gene, which are externally introduced.

[14] The transformant of *Streptomyces lividans* described in [13], wherein the promoter is a promoter of transglutaminase derived from *Streptomyces mobaraensis*.  
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[15] The transformant of *Streptomyces lividans* described in [13] or [14], wherein the terminator is a terminator of transglutaminase derived from *Streptomyces mobaraensis*.

15 [16] The transformant of *Streptomyces lividans* described in any of [13] to [15], wherein the structural gene comprises a sequence set forth in SEQ ID NO: 1 or a sequence obtained by modifying a part of the sequence, the sequence encoding transglutaminase.

20 [17] A transformant of *Streptomyces lividans* comprising a DNA fragment having an externally introduced sequence set forth in SEQ ID NO: 2 or sequence obtained by modifying a part of the sequence, the sequence encoding transglutaminase.

[18] The transformant of *Streptomyces lividans* described in any of [13] to [17],  
25 which is a transformant of *Streptomyces lividans* 3131 or a mutant strain thereof.

[19] A process for producing transglutaminase, comprising the steps of:  
culturing transformant of *Streptomyces lividans* comprising a structural gene of transglutaminase derived from *Streptomyces mobaraensis* and a promoter and a  
30 terminator acting on the structural gene, which are externally introduced, under the conditions where the structural gene can be expressed; and  
collecting the produced transglutaminase.

[20] The process for producing transglutaminase described in [19], wherein the  
35 promoter is a promoter of transglutaminase derived from *Streptomyces mobaraensis*.

[21] The process for producing transglutaminase described in [19] or [20], wherein the terminator is a terminator of transglutaminase derived from *Streptomyces mobaraensis*.

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[22] The process for producing transglutaminase described in any of [19] to [21], wherein the structural gene comprises a sequence set forth in SEQ ID NO: 1 or a sequence obtained by modifying a part of the sequence, the sequence encoding transglutaminase.

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[23] The process for producing transglutaminase described in any of [19] to [21], wherein the transformant of *Streptomyces lividans* comprises a DNA fragment having an externally introduced sequence set forth in SEQ ID NO: 2 or a sequence obtained by modifying a part of the sequence, the sequence encoding transglutaminase.

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[24] The process for producing transglutaminase described in any of [19] to [23], wherein the transformant of *Streptomyces lividans* is a transformant of *Streptomyces lividans* 3131 or a mutant strain thereof.

## 20 BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 shows a method of constructing a shuttle vector pSV1 in Examples.

Fig. 2 shows a method of constructing a secretion expression plasmid pUJ51BD containing a transglutaminase gene.

25 Fig. 3 shows a base sequence of a promoter region of a transglutaminase (BTG) gene determined in Examples.

Fig. 4 shows a base sequence of a promoter region and a part of a structural gene of a transglutaminase (BTG) gene.

Fig. 5 shows a base sequence of a part of a structural gene and a terminator region of a transglutaminase (BTG) gene.

30 Fig 6 is a table summarizing measurement results of the amount of BTG in a culture supernatant of transformants ABL-1 and ABM-1, respectively.

Fig. 7 shows the results of an electrophoresis of the culture supernatant of the transformants ABL-1 and ABM-1 (gel after silver staining). Lanes 1, 2, 3, 4 and 5 apply culture supernatant of *S. lividans* 3131-TS, culture supernatant of ABL-1,

35 purified BTG, culture supernatant of *S. mobaraensis* S-8112, and culture supernatant of

ABM-1, respectively. Lane M applies a molecular weight marker (Pharmacia).

## BEST MODE FOR CARRYING OUT THE INVENTION

Hereinafter, the detail configuration of the present invention will be described.

- 5 The present invention provides a transformant actinomycetes (*Streptomyces mobaraensis* or *Streptomyces lividans*) comprising a structural gene of transglutaminase derived from *Streptomyces mobaraensis*, and a promoter and a terminator acting on the structural gene, which are externally introduced.

- Herein, the kind of structural gene of transglutaminase is not particularly  
10 limited as long as it is derived from *Streptomyces mobaraensis*. For example, a structural gene of transglutaminase carried by *Streptomyces mobaraensis* S-8112 can be used. A specific example of the structural gene can include a DNA consisting of a base sequence set forth in SEQ ID NO: 1. Note here that *Streptomyces mobaraensis* S-8112 is deposited (accession number: FERM P-18980) with the following depositary  
15 agency.

Depositary agency

Name: National Institute of Advanced Industrial Science and Technology,  
International Patent Organism Depositary

- Address: Chuo No. 6, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, 305-8566,  
20 Japan

Deposition date: August 20, 2002

- The structural gene of transglutaminase can be obtained as follows, for example. That is to say, a genomic DNA library of *Streptomyces mobaraensis* is  
25 constructed, and this library is screened by using a probe specific to the structural gene of transglutaminase. Then, an inserted DNA fragment is obtained by restriction enzyme treatment from the selected clone. Note here that the structural gene of transglutaminase can be prepared also by a well-known synthetic method using a PCR method, etc.

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DNA obtained by modifying a part of DNA set forth in SEQ ID NO: 1 (hereinafter, also referred to as "modified DNA") can be also used as the structural gene of the present invention as long as a protein encoded thereby has a transglutaminase activity. Note here that it is preferable that the level of the

transglutaminase activity is as high as possible. For example, it is preferable that the transglutaminase activity is the same level as the transglutaminase activity of a protein encoded by the DNA consisting of the sequence set forth in SEQ ID NO: 1.

5           A specific example of the modified DNA can include a DNA hybridizing to the DNA having the sequence set forth in SEQ ID NO: 1 under stringent conditions and encoding a protein having a transglutaminase activity. Note here that "stringent conditions" herein denote conditions where so-called specific hybrid is formed and non-specific hybrid is not formed. For example, stringent conditions includes  
10   conditions where incubation is carried out by using a hybridization solution (50% formaldehyde, 10 × SSC (0.15 M NaCl, 15 mM sodium citrate, pH 7.0), 5 × Denhardt solution, 1% SDS, 10% dextran sulfate, 10 μg/ml denatured salmon sperm DNA, and 50 mM phosphoric acid buffer (pH 7.5)) at 42°C, followed by washing with 0.1 × SSC and 0.1% SDS at 68°C. Further preferable stringent conditions can include  
15   conditions where a hybridization solution (50% formaldehyde, 5 × SSC (0.15M NaCl, 15 mM sodium citrate, pH 7.0), 1 × Denhardt solution, 1% SDS, 10% dextran sulfate, 10 μg/ml denatured salmon sperm DNA, and 50 mM phosphoric acid buffer (pH 7.5)) is used.

20           Another example of the modified DNA can include DNA consisting of base sequence including substitution, deletion, insertion, addition or inversion of one or plurality of bases in a base sequence set forth in SEQ ID NO: 1 and encoding a protein having a transglutaminase activity. Mutation such as base substitution may be in a plurality of sites. Herein, "plurality" denotes 2 to 40, preferably 2 to 20, and more  
25   preferably 2 to 10 although it is different depending upon kinds or positions of amino acids encoded by bases to be mutated. Note here that such modification includes introduction of a restriction site into 5' end, 3' end or other sites, or addition of a sequence encoding a signal peptide.

30           The above-mentioned modified DNA can be obtained by genetically modifying DNA having a sequence set forth in SEQ ID NO: 1 so that amino acid residues at a certain cite includes substation, deletion, insertion, addition or inversion by the use of, for example, a site specific mutation method. Also, the above-mentioned modified DNA can be obtained by using a well-known mutation  
35   process including processing *Streptomyces mobaraensis* carrying a transglutaminase

gene with ultraviolet light and then isolating the structural gene of the modified transglutaminase, and the like.

Note here that above-mentioned mutation such as substitution, deletion, insertion, addition, inversion, or the like, of bases includes naturally occurring  
5 mutation, for example, mutation based on individual difference of *Streptomyces mobaraensis*.

For example, when naturally existing *Streptomyces mobaraensis* has such a modified DNA, genome (chromosome) DNA is extracted from the strain and processed  
10 with an appropriate restriction enzyme, followed by selecting and isolating DNA that hybridizing under stringent conditions in a screening with the use of the DNA of SEQ ID NO: 1 or a part of the DNA as a probe. Thus, a modified DNA can be obtained.

A promoter acting on the above-mentioned structural gene can be employed.  
15 Preferably, a promoter of transglutaminase derived from *Streptomyces mobaraensis* is employed. Further preferably, a promoter whose origin is the same as that of the structural gene to be used is employed. For example, when the structural gene of transglutaminase is obtained from *Streptomyces mobaraensis* as mentioned above, a DNA fragment including also a promoter region thereof in addition to the structural  
20 gene is obtained and this DNA fragment can be used as the structural gene and the promoter of the present invention.

Also as to the terminator, a terminator acting on the above-mentioned structural gene is employed. Preferably, a terminator of transglutaminase derived  
25 from *Streptomyces mobaraensis* is employed. Further preferably, a terminator whose origin is the same as that of the structural gene to be used is employed. For example, when the structural gene of transglutaminase is obtained from *Streptomyces mobaraensis* as mentioned above, a DNA fragment including also a terminator region thereof in addition to the structural gene is obtained and this DNA fragment can be  
30 used as the structural gene and the terminator of the present invention.

Herein, it is particularly preferable that all of the promoter, structural gene and terminator are derived from the same *Streptomyces mobaraensis*. An example of such embodiment includes the case of using a host *Streptomyces mobaraensis* containing a  
35 DNA fragment having a sequence set forth in SEQ ID NO: 2. Such a DNA fragment



can be prepared so as to include the promoter and terminator regions in addition to the structural gene when the structural gene of transglutaminase is obtained from *Streptomyces mobaraensis* as mentioned above.

Herein, even if the DNA consisting of the sequence set forth in SEQ ID NO: 2  
5 is a DNA obtained by modifying a part of the sequence (modified DNA), such a DNA can be used as long as a protein encoded thereby has a transglutaminase activity. Note here that it is preferable that the level of the transglutaminase activity is as high as possible. For example, it is preferable that the transglutaminase activity is the same level as the transglutaminase activity of protein encoded by the DNA consisting  
10 of the sequence set forth in SEQ ID NO: 2.

As in the case of the DNA of SEQ ID NO: 1, a specific example of the modified DNA can include a DNA hybridizing the DNA having the sequence set forth in SEQ ID NO: 2 under stringent conditions and encoding a protein having the  
15 transglutaminase activity, and DNA consisting of base sequence including substitution, deletion, insertion, addition or inversion of one or a plurality of bases in the base sequence set forth in SEQ ID NO: 2 and encoding a protein having a transglutaminase activity. Besides, the range capable of being modified, a preparing method of modified DNA, and the like, are the same as those of the DNA set forth in SEQ ID  
20 NO: 1.

A transformant of actinomycetes (*Streptomyces mobaraensis* or *Streptomyces lividans*) into which the above-mentioned structural gene, promoter and terminator were externally introduced is produced by transforming a host actinomycete  
25 (*Streptomyces mobaraensis* or *Streptomyces lividans*) with the use of an expression vector containing the structural gene, etc.

As *Streptomyces mobaraensis* to be used for transformation, for example, *Streptomyces mobaraensis* S-8112 (accession number: FERM P-18980) or the mutant strain thereof can be used. On the other hand, as *Streptomyces lividans*, for example,  
30 *Streptomyces lividans* 3131 (ATCC 35287) or the mutant strain thereof can be used. For producing the mutant strain, for example, a well-known method such as ultraviolet irradiation can be used.

For constructing the expression vector, a well-known vector such as  
35 commercially available vector that can be used for transformation of actinomycetes

can be used. For example, an expression vector can be constructed by combining a plasmid, pUC19, pBR322, pBluescript, etc., using *Escherichia coli* as a host and a plasmid, pIJ702, etc. carried by *Streptomyces lividans* 3131, using actinomycetes as a host. Specific examples of the expression plasmid are shown as follows. First of all, a shuttle vector having both replication origin of *Escherichia coli* and replication origin of actinomycetes is constructed by using pUC19 and pIJ702. On the other hand, DNA fragment containing a promoter, a structural gene and a terminator of transglutaminase is isolated from *Streptomyces mobaraensis*, and inserted into an appropriate restriction site of pUC19. Then, pIJ702 and the above-mentioned shuttle vector are used to insert a *tsr* (thiostrepton-resistance) gene into this plasmid. Thus, an expression vector containing a replication origin of *Escherichia coli*,  $Amp^r$  (ampicillin-resistance) gene, a replication origin of actinomycetes and a *tsr* (thiostrepton-resistance) gene, as well as a promoter, a structural gene and a terminator of transglutaminase is obtained.

Restriction enzyme treatment, insertion of a DNA fragment, and the like, for constructing a vector can be carried out by a routine method.

Note here that an expression vector including such a structural gene of transglutaminase derived from *Streptomyces mobaraensis*, and a promoter and terminator acting on the structural gene can be used for transformation of micro-organisms belonging to *Streptomyces* other than *Streptomyces mobaraensis*.

The transformation of actinomycetes (*Streptomyces mobaraensis* or *Streptomyces lividans*) using the expression vector constructed as mentioned above can be carried out by a method of introducing an expression vector into a protoplast host actinomycetes. It is preferable that such a transformation is carried out under the conditions where actinomycetes as a host can be grown. According to the investigation of the present inventors, the efficiency of transformation by such a method is remarkably increased. Other conditions and operation methods, etc. can be carried out by appropriately selecting the conditions and methods employed in a routine method (for example, a method by Turner et al. (Gene, 36, 321-331(1985))). Herein, "the conditions where actinomycetes as a host can be grown" denotes conditions in which a reaction solution contains nutrition necessary for growing actinomycetes. Specific example of such conditions includes conditions in which a reaction solution contains meat extracts, yeast extracts, and/or peptone (including polypeptone, tripeptone, casein peptone, and the like). In order to achieve higher

transformation efficiency, it is further preferable that both step of allowing the host to be a protoplast and a step of introducing a vector into the protoplast host are carried out under such conditions.

The selection of transformant can be carried out by using a selection marker  
5 such as a *tsr* gene, which was incorporated in the expression vector in advance, can be used.

Transglutaminase can be produced by culturing the selected transformant, that is, a host actinomycetes (*Streptomyces mobaraensis* or *Streptomyces lividans*) in which  
10 a structural gene derived from *Streptomyces mobaraensis*, a promoter and a terminator of transglutaminase are externally introduced, under the conditions where the structural gene of transglutaminase can be expressed. As a medium for culturing the transformant, a medium containing a carbon source, a nitrogen source and, inorganic chlorine (inorganic ion) as appropriate can be used. In order to promote the growth of  
15 transformant, a medium, to which vitamin, amino acid, and the like is added, can be used. Examples of the carbon source can include, for example, glucose, starch, dextrin, and the like. Examples of the nitrogen source can include, for example, polypeptone, yeast extracts, meat extracts, and the like. Examples of inorganic chlorine can include dipotassium phosphate, magnesium sulfate, sodium chloride,  
20 potassium chloride, and the like.

The culture temperature for culturing the transformant is, for example, in the range from 15°C to 37°C, and preferably in the range from 25°C to 35°C. Furthermore, pH of the medium is adjusted to, for example, 5.0 to 8.0, and preferably  
25 6.0 to 7.5.

Transglutaminase can be collected from a culture solution in which transformant was cultured for a predetermined time or the cell body. When transglutaminase is collected from the culture solution, transglutaminase can be  
30 obtained by removing insoluble substances via filtration and centrifugation of culture supernatant, followed by isolating and purifying via the combination of salting-out such as ammonium sulfate precipitation, dialysis, various chromatography, and the like. On the other hand, transglutaminase is collected from the cell body, for example, transglutaminase can be obtained by isolating and purifying as mentioned  
35 above after the cell body is crushed by pressure treatment, ultrasonic treatment, etc.

Note here that after the cell body is collected from the culture solution in advance by filtration, centrifugation, and the like, the above-mentioned series of steps (crush, isolation, and purification of the cell body) may be carried out.

## 5 Examples

In Examples of the present invention, unless otherwise indicated, restriction enzymes and other enzymes for gene operation, products of Takara Shuzo Co., Ltd. or TOYOBO CO LTD were used. Note here that the reaction conditions of enzymes, etc. follows the appended instruction manual.

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[Example 1] Obtaining actinomycetes vector pIJ702

*Streptomyces lividans* 3131 (ATCC 35287) containing a plasmid pIJ702 was cultured under the following medium conditions at 30°C for two days.

YEME medium + 0.5% glycine + 50 µg/ml thiostrepton

15	Yeast extracts	3g
	Peptone	5g
	Malt extracts	3g
	Magnesium chloride	1g
	Glucose	10g
20	Saccharose	340g
	Glycine	5g
	50 mg/ml thiostrepton solution (SIGMA: dimethylsulfoxide solution)	
	1 ml / L (pH 7.0)	

25 The 200 ml of cultured medium was centrifuged (12,000g at 4°C for 10 minutes) and the obtained cell body was suspended in 10 ml of solution consisting of 50 mM Tris-HCl (pH 8.0), 10 mM EDTA and 25% Sucrose (hereinafter, referred to as "TE-Sucrose"). Then, 2 ml of TE-Sucrose containing 30 mg/ml of lysozyme (Sigma Aldrich Japan K.K.) and 4 ml of 0.25 mM EDTA were added to the suspension, which  
30 was incubated at 37°C for 30 minutes. After incubation, 2 ml of 20% SDS was added, further 5 ml of 5M NaCl was added and gently stirred, and then incubated at 0°C over night.

Then, 30% polyethylene glycol 6000 was added to supernatant obtained by  
35 centrifugation (100,000 g at 4°C for 40 minutes) so that the final concentration became

10% and incubated at 0°C for 4.5 hours. Thereafter, it was centrifuged (900 g at 4°C for 5 minutes) and the precipitate thus obtained was dissolved in a solution consisting of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and 50 mM NaCl. Then, to this were added 16.8 g of cesium chloride and 1.2 ml of a solution consisting of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (hereinafter, referred to as "TE") in which ethidium bromide had been dissolved to the concentration of 10 mg/ml. The resulting mixture was centrifuged (1,300 g at room temperature for 15 minutes) to remove residues, and then centrifuged (230,000 g at 20°C for 12 hours) again. After centrifugation, under ultraviolet irradiation, a plasmid DNA layer was obtained. Then, extraction with butanol saturated with TE was carried out so as to remove ethidium bromide. The extraction was repeated three times. The obtained plasmid DNA solution was subjected to dialysis over night at 4°C using TE as an external dialysis solution. Then, extraction treatment was carried out once with TE-saturated phenol and twice with chloroform isoamyl alcohol. To this were added 1/10 volume of 3 M sodium acetate (pH 5.2) solution and 2 volumes of ethanol. After standing for 30 minutes at -80°C, the resulting mixture was centrifuged (12,000 g at 4°C for 15 minutes) so as to collect precipitate. The precipitate was washed with 70% ethanol and dried. This was dissolved in 200 µl of TE. By the above-mentioned operation, the final DNA amount was about 10 µg.

20

[Example 2] Obtaining thiostrepton sensitive strain from *Streptomyces lividans* 3131 (ATCC 35287) carrying pIJ702

*Streptomyces lividans* 3131 (ATCC 35287) containing pIJ702 was cultured at 30°C for seven days using a YEME medium. Then, a culture solution was diluted to 10<sup>5</sup> to 10<sup>9</sup> times with the YEME medium. A 100 µl each of the diluted solution was inoculated on each of five plates containing a YEME agar medium (YEME medium supplemented with 1.5% agar). After culturing at 30°C for a week, the cultured medium was replicated on the YEME agar medium containing 200 µg/ml of thiostrepton using RepliPlate™ Colony Transfer Pad (Takara Shuzo Co., Ltd.), and cultured again at 30°C for a week. A strain, from which the plasmid pIJ702 was dropped to become a thiostrepton-sensitive strain, was selected to obtain *Streptomyces lividans* 3131-TS. This strain was used as a host for later transformation.

[Example 3] Obtaining shuttle vector pSV1

Shuttle vector pSV1 was constructed by the method shown in Fig. 1. First of

all, a DNA fragment obtained by digesting *E. coli* vector pUC19 (Takara Shuzo Co., Ltd.) with a restriction enzyme *Bam* HI and a DNA fragment containing *tsr* obtained by digesting actinomycetes vector pIJ702 with *Bcl* I (Takara Shuzo Co., Ltd.) were prepared. These DNA fragments were ligated to each other by using DNA Ligation Kit (Takara Shuzo Co., Ltd.) to produce pUCTSR. Then, a DNA fragment (long  
5 fragment) obtained by digesting pUCTSR with *Kpn* I and *Cla* I (Takara Shuzo Co., Ltd.) and a DNA fragment (short fragment) obtained by digesting pIJ702 with *Kpn* I and *Cla* I (Takara Shuzo Co., Ltd.) were prepared, and these DNA fragments were ligated to each other by using DNA Ligation Kit (Takara Shuzo Co., Ltd.) to transform  
10 to an *E. coli* DH5 strain (TOYOBOKO Co., Ltd.). A plasmid was obtained by ligating pUC19 fragment and pIJ702 fragment which are carried by the thus obtained transformant to obtain a shuttle vector pSV1. The shuttle vector pSV1 was used for the later operation.

15 [Example 4] Manufacture of transglutaminase secretion expression plasmid pUJ51BD

A secretion expression plasmid pUJ51BD containing transglutaminase gene was constructed by the method shown in Fig. 2. First of all, a plasmid pBTG-BB was manufactured by cutting out about 6.6 kb of *Bgl* II-*Bam* HI fragment from a phage DNA ( $\lambda$ BTG, Japanese Patent Unexamined Publication No. 5-199883) containing a  
20 transglutaminase (hereinafter, also referred to as "BTG") gene *Bam* HI fragment isolated from *Streptomyces mobaraensis* S-8112 (Accession number: FIRM P-18980) and by inserting the fragment into a *Bam* HI site of pUC19. A 3' unnecessary region was deleted from pBTG-BB by using Kilo-Sequence Deletion Kit (Takara Shuzo Co., Ltd.) to manufacture a plasmid pU51B containing about 3.9 kb of BTG gene. Then, a  
25 plasmid pUJ51B was manufactured by inserting *Pst* I-digested DNA fragment of actinomycetes vector pIJ702 into a *Pst* I site of pU51B. By cutting out a *Xba* I-*Cla* I fragment from pUJ51B and substituting it by a *Xba* I-*Cla* I fragment of *E. coli*-actinomycetes shuttle vector pSV1 obtained in Example 3, a BTG secretion expression plasmid pUJ51BD from which a *mel* (tyrosinase) gene derived from pIJ702  
30 was removed was constructed.

[Example 5] Analysis of base sequence of promoter region

A synthetic primer BB-23 [Invitrogen Corporation]  
5'-ACACCGCACTCATAGTGGCG-3' (SEQ ID NO: 3) for analyzing a base sequence  
35 was synthesized from a sequence (SEQ ID NO: 1) of a structural gene of BTG. The

base sequence of plasmid pBTG-BB was analyzed by using a primer BB-23 from 3' end of the promoter region and by using a M13-RV (Takara Shuzo Co., Ltd.) from 5' end of the promoter region. From the results of analysis of resultant base sequence, further synthesized primer BB-19 5'-TCCGTGCGAGTGGAAGAACG-3' (SEQ ID NO: 4) and SP6-20 5'-GACGGCCTCCGAATAAC-3' (SEQ ID NO: 5) were synthesized. By the Primer walking method using these sequences, entire base sequence of about 700 bp of promoter region was determined (Fig. 3). Similarly, the base sequence of about 500 bp of terminator region was determined by the Primer walking method using synthetic primers SP6-32 5'-ATGTCGAGGGACAGGAAC-3' (SEQ ID NO: 6) and SP6-36 5'-CACCACGAAAGTCGCTAC-3' (SEQ ID NO: 7). As a result, a base sequence (SEQ ID NO: 2) consisting of a promoter region, a structural gene and a terminator region was identified (Figs. 4 and 5). For Sequence reaction, BigDye™ Terminator Cycle Sequencing FS Ready Kit (Applied Biosystems) was used, and for analysis, ABI PRISM 310 Sequencer (Applied Biosystems) was used.

[Example 6] Preparation of *Streptomyces lividans* 3131-TS protoplast

*Streptomyces lividans* 3131-TS which had been obtained in Example 2 was cultured using a YEME medium (0.5% glycine) at 30°C for two days. The 200 ml of cultured medium was centrifuged (1,300 g at room temperature for 10 minutes). The obtained cell body was suspended in 72 ml of 0.35 M saccharose solution. Then, this suspension was centrifuged (1,300 g at room temperature for 10 minutes) and the cell body was suspended again in 60 ml of buffer solution P containing 1 mg/ml of lysozyme (Sigma Aldrich Japan K.K.) and the suspension was incubated at 30°C for 2.5 hours. The suspension after incubation was filtrated with absorbent cotton so as to remove residues. Then, the resultant filtrate was centrifuged (1,300 g at room temperature for 10 minutes) and the sediment was washed with 25 ml of buffer solution P. This washing was repeated twice and precipitation was suspended in 1 ml of buffer solution P to obtain a protoplast suspension.

Buffer solution P

TES [N-Tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid]	5.73g
Saccharose	103g
Magnesium chloride	2.03g
Potassium sulfate	0.5g

Calcium chloride	3.68g
Trace element solution	2 ml /L (pH 7.4)

Note here that 1% monobasic potassium phosphate solution was prepared separately, which was added in the amount of 1 ml per 100 ml buffer solution P  
 5 immediate before use.

Trace element solution

	Zinc chloride	40 mg
	Ferric chloride	200 mg
	Cupric chloride	10 mg
10	Manganese chloride	10 mg
	Sodium tetraborate	10 mg
	Ammonium molybdate	10 mg /L

[Example 7] Transformation of *Streptomyces lividans* 3131-TS

15	Each of the following solutions was mixed to the total amount	140 $\mu$ l.
	DNA solution of BTG secretion expression plasmid pUJ51D	20 $\mu$ l
	<i>Streptomyces lividans</i> 3131-TS protoplast	100 $\mu$ l
	0.35 M saccharose solution	20 $\mu$ l

Then, 1.5 ml of buffer solution P containing 20% polyethylene glycol 1000  
 20 was added to the reaction mixture and mixed gently by pipetting. The resulting mixture was allowed to remain still for two minutes at room temperature. The mixture was centrifuged (1,700 g at room temperature for 10 minutes) to collect precipitate. Protoplast obtained as precipitate was washed twice with buffer solution P. The washed precipitate pellet was suspended in 1 ml of buffer solution P, and 200  
 25  $\mu$ l each of the suspension was applied on an R-2 medium.

On the other hand, R-2/A and R-2/B as shown below were prepared.

R-2/A

	Potassium Sulfate	0.5 g
	Magnesium Chloride	20.2 g
30	Calcium Chloride	5.9 g
	Glucose	20.0 g
	Proline	6.0 g
	Casamino Acid	0.2 g
	Trace Element Solution	4 ml
35	Agar	44.0 g /L



R-2/B

	TES	11.5 g
	Yeast Extracts	10.0 g
5	Sucrose	203 g /L (pH 7.4)

When a plate medium was produced, R-2/A and R-2/B were mixed and 1%  $\text{KH}_2\text{PO}_4$  solution further was mixed in the amount of 1 ml per 200 ml final volume. The mixture was incubated at 30°C for 18 hours. The surface of the plate medium was covered with 1 ml of buffer solution P consisting of 200 µg/ml of thiostrepton and  
 10 400 µg/ml of tyrosine. The resulting plates were further incubated at 30°C for 7 days to obtain thiostrepton-resistant transformant (ABL-1).

[Example 8] Preparation of protoplast of *Streptomyces mobaraensis*

As preculture, *Streptomyces mobaraensis* S-8112 was cultured by using a  
 15 YEME medium + 1% glucose + 25 mM magnesium chloride (pH 7.0) at 27°C for three days. Then, the 0.5% precultured culture solution was inoculated on a YEME medium + 1% glucose + 4 mM magnesium chloride + glycine (pH 7.0) and cultured at 27°C for three days. The cultured medium was centrifuged (3,000 g at 4°C for 10 minutes) and the wet weight of the obtained cell body was measured. 0.6 g of wet  
 20 cell body was washed with 10 ml of 0.3 M saccharose solution and centrifuged again (3,000 g at 4°C for 10 minutes). The cell body was suspended in 4 ml of buffer solution BS-L consisting of 0.4 mg/ml of lysozyme (Roche Japan) and 0.1 mg/ml of Achromopeptidase (Wako Pure Chemical Industries, Ltd.) and gently stirred at 30°C for 30 minutes. Then, 5 ml of buffer solution BS-P was added on ice and filtrated  
 25 with absorbent cotton while washing with 3 ml of buffer solution BS-P so as to carry out filtration. The obtained suspension was centrifuged (1,500 g at 4°C for 5 minutes), suspended in 10 ml of buffer solution BS-P, and then centrifuged again (1,500 g at 4°C for 5 minutes). The obtained precipitate was suspended in 0.5 ml of buffer solution BS-P to obtain a protoplast suspension, which was used for the below  
 30 mentioned transformation.

Buffer solution BS-L

	10% meat extracts	10 ml
	Yeast extracts.	1 g
35	Peptone	2 g

18

Glucose	10 g
Saccharose	171.15 g
Calcium chloride	0.277 g
Magnesium chloride	0.508 g /L (pH 7.0)

5

Buffer solution BS-P

10% meat extracts	10 ml
Yeast extracts	1 g
Peptone	2 g
10 Glucose	10 g
Saccharose	171.15 g
Calcium chloride	2.77 g
Magnesium chloride	2.03 g /L (pH 7.0)

15 [Example 9] Transformation of *Streptomyces mobaraensis*

After 100  $\mu$ l of protoplast suspension (protoplast concentration:  $1 \times 10^9$ /ml) obtained in Example 8 and 10  $\mu$ l of 2M saccharose solution containing 1  $\mu$ g of BTG secretion expression plasmid pUJ51D obtained in Example 4 were mixed, immediately the mixture was allowed to remain still for 10 seconds. Then, after 0.5 ml of buffer solution P containing 25% polyethylene glycol 1000 (Sigma Aldrich Japan K.K.) was added to the mixture, the mixture was immediately allowed to remain still on ice for one minute. Then, after 2 ml of buffer solution BS-P was added to the mixture, the mixture was immediately centrifuged (1,500 g at 4°C for 10 minutes). The resultant precipitate was suspended in 0.5 ml of buffer solution BS-P and 0.1 ml each of the suspension was dispensed on a SBS agar medium to superpose it by using 3 ml of SBS agar medium while stirring. After removing the cover, the agar was dried for exactly two hours, and then cultured for exactly 24 hours at 27°C. Furthermore, 3 ml of SBS soft agar medium containing 200  $\mu$ g/ml of thiostrepton was superposed and cultured at 27°C. The transformant obtained after the above-mentioned operation was defined as

30 ABM-1.

SBS agar medium (pH 7.0)

Meat extracts	10 g
Yeast extracts	7.5 g
35 Tryptone peptone	1 g

19

	Glucose	10 g
	Saccharose	308.07 g
	Agar	25g /L (pH 7.0)
	<u>SBS soft agar medium (pH 7.0)</u>	
5	Meat extracts	10 g
	Yeast extracts	7.5 g
	Tryptone peptone	1 g
	Glucose	10 g
	Saccharose	308.07 g
10	Sea Plaque agarose	25g /L (pH 7.0)

[Example 10] Culture of transformant in which BTG gene was incorporated  
 Transformant ABL-1 obtained in Example 7 and transformant ABM-1  
 obtained in Example 9 were cultured at 30°C for 7 days under the following  
 15 conditions, respectively.

	Poly peptone	20 g
	Soluble starch	20 g
	Yeast extracts	2 g
	Dipotassium phosphate	2 g
20	Magnesium sulfate	1 g
	Adekanol LG126	0.5 g
	50 mg/ml thiostrepton solution	0.5ml /L (pH 7.0)

The medium cultured under the above-mentioned conditions was centrifuged  
 25 (12,000g at 4°C for 10 minutes) and the obtained supernatant was subjected to ELISA  
 method.

[Example 11] Measurement of production amount of BTG (ELISA method)  
 From the culture supernatant of *Streptomyces mobaraensis* S-8112, the  
 30 amount of BTG in the culture supernatant obtained in Example 10 was measured by  
 the ELISA method using an anti-BTG antibody produced by immunizing a rabbit by  
 using as an antigen purified BTG obtained in affinity chromatography using Blue  
 Sepharose CL-6B (Pharmacia).

First of all, to each well of a 96-well micro plate, 100 µl each of anti-BTG  
 35 antibody solution which had been diluted with PBS buffer was dispended, incubated at

37°C for one hour, and immobilized on the plate. After removing the antibody solution, each well was washed with 200 µl of 10 times diluted solution of Block Ace (Dainippon Pharmaceutical Co., Ltd.) containing 0.1% Tween20 (hereinafter, referred to as “washing solution”). Washing was carried out successively three times. Then, 200 µl of 4 times diluted solution of Block Ace was dispensed in each well and incubated at 37°C for one hour so as to be blocked. After removing 4 times diluted solution of Block Ace, each well was washed with the washing solution three times. Then, measurement sample, i.e., culture supernatant (culture supernatant of ABL-1 or ABM-1) was appropriately diluted with 10 times diluted solution of Block Ace and 50 µl each of the diluted solution was dispensed in each well and incubated at 37°C for one hour. Note here that as standards, purified BTG which had been diluted with 10 times diluted solution of Block Ace were prepared at different concentrations. Similar to the measurement samples, the standards were also added to the well which had been subjected to blocking treatment.

After a sample solution was removed from each well, each well was washed with washing solution three times. Then, 100 µl of 10 times diluted solution of Block Ace containing Fab'-HRP obtained by cross-linking horseradish peroxidase (HRP) to Fab' fragment of anti-BTG antibody was dispensed in each well and incubated at 37°C for one hour. Then, after Fab'-HRP solution was removed, each well was washed with washing solution three times. Then, 150 µl each of 50 mM sodium citrate solution (pH 4.5) containing 0.04% orthophenylenediamine (*o*-PDA) and 0.42% peroxide solution was dispensed in each well and incubated at 37°C to allow *o*-PDA and peroxide to react with HRP for coloring. Exactly after 20 minutes from the start of the reaction, 50 µl each of 3M sulfuric acid solution as a stop solution of the reaction was dispensed in each well, and the absorbance at 492 nm was measured. Then, by using a calibration curve calculated from the absorbance of standard, the amount of BTG in each culture supernatant (ABL-1 or ABM-1) was calculated. Fig. 6 is a table showing BTG amount per each culture supernatant. As shown in this table, BTG was produced in the amount of 0.7 g/L for ABL-1 and 0.5 g/L for ABM-1. When compared with the already reported productivity (40 mg /L to 50 mg/L), ten time or more of ABL-1 and about 10 times of AMB-1 were obtained. Thus, BTG productivity with extremely high efficiency was confirmed.

#### Buffer solution PBS

35	Sodium chloride	8.0g
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Dipotassium phosphate	1.1g
Potassium chloride	0.2g
Monobasic potassium phosphate	0.2g /L (pH 7.4)

#### 5 [Example 12] SDS-PAGE

The culture supernatants of transformant ABL-1 and transformant ABM-1 are mixed with 2xSDS buffer solution for electrophoresis at the ratio of 1 : 1, repeatedly and allowed them to be samples for SDS-PAGE. Note here that as the control, a culture supernatant of *Streptomyces lividans* 3131-TS, purified BTG diluted with a  
 10 buffer solution, and a culture supernatant of *Streptomyces mobaraensis* S-8112 were prepared. Each sample was subjected to SDS-PAGE using 12.5% gel. For SDS-PAGE, Fast-System (Pharmacia Biotech) was used and for staining, silver staining was carried out. Fig. 7 shows photographs of gels after silver staining. In Fig. 7, lanes 1, 2, 3, 4 and 5 apply culture supernatant of *S. lividans* 3131-TS, culture  
 15 supernatant of ABL-1, purified BTG, culture supernatant of *S. mobaraensis* S-8112, and culture supernatant of ABM-1, respectively. Lane M applies a molecular weight marker (Pharmacia).

As shown in Fig. 7, in ABL-1 (lane 2) and ABM-1 (lane 5), clear bands were observed in substantially the same positions in the purified BTG (lane 3), showing that  
 20 BTG was contained at high concentration. On the other hand, in the culture supernatant of *Streptomyces lividans* (*S. lividans*) 3131-TS (lane 1) and the culture supernatant of *Streptomyces mobaraensis* (*S. mobaraensis*) S-8112 (lane 4), bands showing the purified BTG are not observed. This shows that in ABL-1 and ABM-1, BTG is specifically produced.

25

#### Buffer solution 2xSDS

Tris-HCl	2.42 g
EDTA	0.744 g
SDS	50 g
30 $\beta$ -mercaptoethanol	100 ml /(pH8.0)

The present invention is not limited to the description of the above embodiments. A variety of modifications, which are within the scopes of the following claims and which are achieved easily by a person skilled in the art, are  
 35 included in the present invention.

Hereinafter, the following matters are disclosed.

- (1) A process for producing transglutaminase, comprising the steps of:  
transforming a host *Streptomyces mobaraensis* by using a vector containing a  
5 structural gene of transglutaminase derived from *Streptomyces mobaraensis*, and a  
promoter and a terminator acting on the structural gene;  
culturing the transformant under the conditions where the structural gene can  
be expressed; and  
collecting the produced transglutaminase.  
10
- (2) The process for producing transglutaminase described in (1), wherein the  
step of transforming is carried out under the conditions where the host *Streptomyces*  
*mobaraensis* can be grown.
- 15 (3) The process for producing transglutaminase described in (1) or (2),  
wherein the vector comprises a plasmid obtained by modifying a plasmid pIJ702.
- (4) The process for producing transglutaminase described in any of (1) to (3),  
wherein the promoter is a promoter of transglutaminase derived from *Streptomyces*  
20 *mobaraensis*.
- (5) The process for producing transglutaminase described in any of (1) to (4),  
wherein the terminator is a terminator of transglutaminase derived from *Streptomyces*  
*mobaraensis*.  
25
- (6) The process for producing transglutaminase described in any of (1) to (5),  
wherein the host *Streptomyces mobaraensis* is *Streptomyces mobaraensis* S-8112 or a  
mutant strain thereof.
- 30 (7) The process for producing transglutaminase described in any of (1) to (6),  
wherein the structural gene comprises a sequence set forth in SEQ ID NO: 1 or a  
sequence obtained by modifying a part of the sequence, the sequence encoding  
transglutaminase.
- 35 (8) The process for producing transglutaminase described in any of (1) to (6),

wherein the transformant comprises a DNA fragment having an externally introduced sequence set forth in SEQ ID NO: 2 or sequence obtained by modifying a part of the sequence, the sequence encoding transglutaminase.

5           (11) A process for producing transglutaminase, comprising the steps of:  
transforming a host *Streptomyces lividans* by using a vector containing a  
structural gene of transglutaminase derived from *Streptomyces mobaraensis*, and a  
promoter and a terminator acting on the structural gene;  
culturing the transformant under the conditions where the structural gene can  
10 be expressed; and  
collecting the produced transglutaminase.

(12) The process for producing transglutaminase described in (11), wherein  
the step of transforming is carried out under the conditions where the host  
15 *Streptomyces lividans* can be grown.

(13) The process for producing transglutaminase described in (11) or (12),  
wherein the vector comprises a plasmid obtained by modifying a plasmid pIJ702.

20           (14) The process for producing transglutaminase described in any of (11) to  
(13), wherein the promoter is a promoter of transglutaminase derived from  
*Streptomyces mobaraensis*.

(15) The process for producing transglutaminase described in any of (11) to  
25 (14), wherein the terminator is a terminator of transglutaminase derived from  
*Streptomyces mobaraensis*.

(16) The process for producing transglutaminase described in any of (11) to  
(15), wherein the host *Streptomyces lividans* is *Streptomyces lividans* 3131 or a mutant  
30 strain thereof.

(17) The process for producing transglutaminase described in any of (11) to  
(16), wherein the structural gene comprises a sequence set forth in SEQ ID NO: 1 or a  
sequence obtained by modifying a part of the sequence, the sequence encoding  
35 transglutaminase.

(18) The process for producing transglutaminase described in any of (11) to (16), wherein the transformant comprises a DNA fragment having an externally introduced sequence set forth in SEQ ID NO: 2 or sequence obtained by modifying a part of the sequence, the sequence encoding transglutaminase.

(21) An expression vector comprising a structural gene of transglutaminase derived from *Streptomyces mobaraensis*, and a promoter and a terminator acting on the structural gene.

10

(22) The expression vector described in any of (21), wherein the promoter is a promoter of transglutaminase derived from *Streptomyces mobaraensis*.

(23) The expression vector described in any of (21) or (22), wherein the terminator is a terminator of transglutaminase derived from *Streptomyces mobaraensis*.

15

(24) The expression vector described in any of (21) to (23), wherein the structural gene comprises a sequence set forth in SEQ ID NO: 1 or a sequence obtained by modifying a part of the sequence, the sequence encoding transglutaminase.

20

(25) An expression vector having a sequence comprising a sequence set forth in SEQ ID NO: 2 or a sequence obtained by modifying a part of the sequence, the sequence encoding transglutaminase.

(26) A micro-organism belonging to *Streptomyces*, which was transformed by using any of expression vectors of (21) to (25).

25

(31) A process for transforming actinomycetes, comprising the steps of :  
allowing a host actinomycetes to be a protoplast under the conditions where it can be grown; and

30

introducing an expression vector comprising a structural gene of transglutaminase derived from *Streptomyces mobaraensis*, and a promoter and a terminator acting on the structural gene; into host actinomycetes under the conditions where the host actinomycetes can be grown.

35



(32) The process for transforming actinomycetes described in (31), the host actinomycetes is either *Streptomyces mobaraensis* or *Streptomyces lividans*.

## **INDUSTRIAL APPLICABILITY**

5           The present invention provides an actinomycetes capable of producing transglutaminase with extremely high productivity. The use of the actinomycetes makes it possible to produce transglutaminase at a high efficiency.